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Flightless brown kiwis of New Zealand possess extremely subdivided population structure and cryptic species like small mammals

(mtDNA sequencing/allozymes)

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ABSTRACT **Using allozymes and mtDNA sequences from the cytochrome *b* gene, we report that the brown kiwi has the highest levels of genetic structuring observed in birds. Moreover, the mtDNA sequences are, with two minor exceptions, diagnostic genetic markers for each population investigated, even though they are among the more slowly evolving coding regions in this genome. A major unexpected finding was the concordant split in molecular phylogenies between brown kiwis in the southern South Island and elsewhere in New Zealand. This basic phylogeographic boundary halfway down the South Island coincides with a fixed allele difference in the *Hb* nuclear locus and strongly suggests that two morphologically cryptic species are currently merged under one polytypic species. This is another striking example of how molecular genetic assays can detect phylogenetic discontinuities that are not reflected in traditional morphologically based taxonomies. However, reanalysis of the morphological characters by using phylogenetic methods revealed that the reason for this discordance is that most are primitive and thus are phylogenetically uninformative. Shared-derived morphological characters support the same relationships evident in the molecular phylogenies and, in concert with the molecular data, suggest that as brown kiwis colonized northward from the southern South Island, they retained many primitive characters that confounded earlier systematists. Strong subdivided population structure and cryptic species in brown kiwis seem to have evolved relatively recently as a consequence of Pleistocene range disjunctions, low dispersal power, and genetic drift in small populations.**

Molecular genetic assays of intraspecific variation over the past 20 years have established the orthodox view that bird species exhibit only weak population structure relative to most other vertebrates (1–3). This finding is expected under population genetic theory because increased dispersal capability via flight should translate into greater amounts of realized gene flow than in less vagile vertebrates (4). However, recent refinements in molecular methods and the development of companion genetic theory and analytical methods have begun to challenge the near exclusivity of this generalization and have spawned a vigorous reexamination of population structure and phylogeography of bird species. In particular, the application of direct sequencing of maternally inherited mtDNA has provided a rich source of genetic markers that can detect recently evolved population structure and help reconstruct intraspecific phylogenies of matriarchal lineages.

For example, variation in part of the sequence coding for the cytochrome *b* gene and particularly in the noncoding control region of mtDNA in dunlins (*Calidris alpina*) revealed pronounced global population structuring in this long-distance migrant shorebird (5). Thus, we might predict that flightless species of birds with low dispersal capability could potentially exhibit extreme population structure. The flightless kiwis of New Zealand are ideal candidate species for study in this context because they exhibit a number of extraordinary biological features that could impact on their intraspecific phylogeography. First, with their nocturnal habits, use of burrows, hairlike feathers, facial bristles, two functional ovaries, well-developed sense of smell, lower body temperature, near absence of wings, and consequent low dispersal power (6), the kiwis are more like small mammals than birds (7). Second, even the brown kiwi (*Apteryx australis*), the most abundant and widespread of the three species of kiwis, has suffered severe reductions in numbers from hunting, habitat destruction, and the introduction of mammalian predators. Formerly widespread over much of New Zealand (6), it is now largely restricted to small, disjunct populations that are unlikely to be exchanging genes and are prone to local extinctions. Like species of small mammals fragmented into small disjunct populations, the brown kiwi could be expected to have pronounced geographic population structure and possibly to harbor morphologically cryptic species of direct relevance to their conservation management (8).

Phylogeographic subdivision has always been implicit in the taxonomy of brown kiwis. Following their discovery last century, the North and South Island populations were eventually recognized in 1850 as separate species (*Apteryx mantelli* and *A. australis*, respectively) (9), on the basis of morphological characters, such as length of facial bristles, size of body parts, plumage color, wing quills, and tarsus scutellation (10, 11). However, subsequent research with additional specimens suggested that the only valid character separating them was the stiffer feather tips of the North Island species. Thus, in 1899, Rothschild (12) relegated the North and South Island brown kiwis to subspecies (*Apteryx australis mantelli* and *Apteryx australis australis*, respectively) and also recognized the Stewart Island population as *Apteryx australis lawryi*. This taxonomic assignment is now widely accepted in current classifications (13).

The recent discovery of differences in feather lice (14, 15), blood proteins (16), and possibly calls among populations of brown kiwis stimulated us to conduct a molecular genetic analysis of blood samples obtained from locales covering their presently known range (Fig. 1). The objectives of the analysis were to determine the degree of population structuring in this flightless species and to test the adequacy of the morpholog-

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Abbreviation: UPGMA, unweighted pair-group method using arithmetic averages.

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FIG. 1. Distribution of extant regional populations of brown kiwis in New Zealand. Locales sampled for this study are shaded. Sample sizes analyzed for allozymes and mtDNA, respectively, are shown in parentheses as follows: Little Barrier Island (8, 10), Whakatane (2, 2), Wairoa (3, 2), Waitangi (36, 4), New Plymouth (5, 5), Okarito (10, 10), Haast (3, 8), Fiordland (5, 7), Stewart Island (36, 10), *Apteryx haasti* (8, 2), and *Apteryx oweni* (33, 1).

ically derived taxonomy with independent molecular phylogenies.[‖]

MATERIALS AND METHODS

Sampling Locales. Blood samples (1–2 ml) were collected in EDTA from live-caught birds at locales in New Zealand where brown kiwis still occur and where the terrain is possible to work. Locations of sample sites are shown in Fig. 1, and sample sizes are indicated in the legend. Blood samples from little spotted kiwis (*A. oweni*) and greated spotted kiwis (*A. haasti*) were also obtained for outgroup analysis.

PCR Amplification and Sequencing of mtDNA. Total DNA was extracted from whole blood with standard procedures (17), and ≈0.5 μg was subjected to 35 cycles of amplification in a thermal cycler (Perkin–Elmer/Cetus) to produce double-stranded mtDNA. Amplifications were performed in 25-μl reaction volumes with 1 unit of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). Two contiguous segments of the mitochondrial cytochrome *b* gene were amplified by using the universal primers in ref. 18 and the following primer pair: L15212 (5′-GGACGAGGCTTTTACTACGGCTC-3′) and H15649 (5′-TTGCTGGGGTGAAGTTTTCTGGGTC-3′). L and H refer to the light and heavy strands, respectively, and the numbers correspond to the nt position at the 3′ end of the primer in the chicken mtDNA sequence. These primers were used to amplify 654 bp of internal sequence by using the temperature profile in ref. 5. PCR products were purified with (Bio 101) and subjected to double-stranded sequencing with a Sequenase 2.0 kit (United States Biochemical) and deoxyadenosine[α-[^{35}S]thio]triphosphate. All specimens were sequenced twice with the four primers, and a minimum of 70% overlap between strands was achieved. There were no ambiguous sites.

Allozymes. Starch gel electrophoresis was carried out to score the genotypes at 29 presumptive loci expressed in blood for a larger set of samples from the same locales from which mtDNA sequence data were obtained (Table 1). Gels were run overnight at 4°C, sliced, and stained to visualize protein products following standard procedures (19, 20). Allele frequencies were calculated for each population and used for analysis of population structure and for phylogeny estimation.

Estimation of Population Structure and Molecular Phylogenies. The extent of population structure in the brown kiwi was estimated for the mtDNA sequences with a measure of mutational divergence (γ) as in ref. 21. For the allozyme data, we used Wright's traditional F_{ST} statistic.

Genealogical relationships among mtDNA haplotypes were estimated with the neighbor-joining method in MEGA (22), with maximum likelihood in PHYLIP v. 3.5 (23), and with maximum parsimony in the computer package PAUP v. 3.1.1 (24). In the parsimony analysis, a strict consensus tree was derived from the four equally parsimonious trees computed with the branch and bound option. Allozyme trees depicting relationships among populations were computed with the unweighted pair-group method using arithmetic averages (UPGMA) method on Nei's genetic distance. Trees were also computed with the Fitch–Margoliash (FITCH) and maximum likelihood (CONTML) methods in PHYLIP.

RESULTS

Cytochrome *b* Sequence Variation. Twenty-one haplotypes occurred in the 60 brown kiwis we sequenced (Fig. 2). In 654 bp of sequence, 42 (6.4%) variable sites were found, of which 41 were transitions and 1 was a transversion. The transversion at position 648 distinguishes South Island populations, which possess a cytosine, from North Island populations, which have an adenine substitution at this position. As with the brown kiwis of the South Island, the outgroup reference sequence from the great spotted kiwi (*A. haasti*) possesses a cytosine. The two species of spotted kiwis have an additional transversion relative to the brown kiwi at position 300. Thirty-six substitutions occur at third positions in codons, two occur at second positions, and four occur at first positions. All third-position substitutions are silent, whereas half the changes at first and second positions cause amino acid replacements. These replacements occur only in South Island brown kiwis; first-position substitutions (positions 196 and 478 in the sequence) cause a leucine for phenylalanine substitution in Okarito birds and an alanine for threonine substitution in all South Island birds, respectively. In Stewart Island birds, a second-position substitution (position 401) causes a valine for alanine replacement.

Allozyme Variation. Only 3 of the 29 loci that we examined are polymorphic in brown kiwi populations, attesting to their low within-population variability (Table 2). Both the Stewart Island and neighboring Fiordland populations in southern New

Table 1. Population genetic structure in brown kiwis

Comparison	Cytochrome *b* γ*	Allozymes F_{ST}†
All populations	0.987	0.615
North Island and Okarito vs. South Island	1.000	0.542
Within North Island and Okarito	0.965	0.215
Within southern South Island	1.000	0.525

*$\gamma = 1 - I_B/I_W$, where I_B and I_W are the probabilities of identity of sequence variants between and within populations, respectively.
†$F_{ST} = H_T - H_S/H_T$, where H_T and H_S are total heterozygosity and average subpopulation heterozygosity, respectively.

‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U28695–U28717).

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                 0000001111111122222223333344444444445566666
                 1157890112238903577880033600445678835002345
                 5675412163629617503280906618473886972041380
1.  A.haasti     CTGTCCACTACATTCCTAGATCTTGTCACCAAATCACATCCC
2.  Whaka1       T.........GC......G.A...C.....GGC.GT...TA
3.  Whaka2       T.........G.......G.A...C.....GGC.GT...TA
4.  Wairoa1      T.........GC........A...C.....GGC.GT...TA
5.  Wairoa2      T.........GC........A...C......GC.GT...TA
6.  Waitangi     T...........C........AC..C......GC.GT...TA
7.  Waitang2     T...G.......C........AC..C......GC..T...TA
8.  NewPlym1     TC.........GC........AC..C......GC.G....TA
9.  LitBarr1     TC.........GC.....A..A...C......GC.GT...TA
10. LitBarr2     TC.........GC........A...C......GC.G....TA
11. LitBarr3     TC.........GC........A...C......GC.GT...TA
12. Okarito1     T..........GCCTT..A..A...C.G....GCT.TG.TT.
13. Okarito2     T..........GCC....A..A...C.G....GCT.TG.TT.
14. Haast1       .........GTG....CG..CT.CA..G.....C..T.C...
15. Fiordl1      ..AC.T...G.G....C...CT.C...G..G..C..T...T.
16. Fiordl2      ......G..GTG....C...CT.CA..GT....C..T...T.
17. Stewart1     .........G.GC........T.C..TG.T......T...T.
18. Stewart2     ..AC...TC..G....C...CT.C..TG.T......T...T.
19. Stewart3     ...........GC........T.C..TG.T......T...T.
20. Stewart4     ..AC....C..G....C...CT.C..TG.T......T...T.
21. Stewart5     ...........GC...C...CT.C..TG.T......T...T.
22. Stewart6     ..AC....C..G....C...CT.C..TG.T...C..T...T.
23. A.oweni      ..............................G.........
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FIG. 2. Mitochondrial DNA sequence variation from 654 bp of the cytochrome *b* gene in 60 brown kiwis. Only the 42 variable sites are shown, and their sequence location is indicated by the three digits at the top. Sequence identity with the great spotted kiwi (*A. haasti*) is indicated by dots, and variation at these sites is shown for the little spotted kiwi (*A. oweni*) in the bottom row. Spotted kiwis have 14 additional fixed substitutions from brown kiwis at sites not shown here.

Zealand are monomorphic at all loci, the Haast population is polymorphic at *Ak-1*, and the Okarito and North Island populations are polymorphic at *Ak-1* and *Ldh-2*. The three variable loci exhibit pronounced allelic shifts among populations. At *Hb*, there is a fixed allele difference between the North Island and Okarito populations on the one hand and the remaining South Island populations on the other. The *C* allele at *Ak-1* is at high frequency in the Haast population but occurs nowhere else, and the *C* allele at *Ldh-2* is common in the Okarito population, rare in the North Island populations, and absent in the southern South Island populations.

Phylogeography. Molecular phylogenies based on sequences from the mitochondrial cytochrome *b* gene or allozymes have nearly identical topologies (Fig. 3). They feature a primary division between birds from the North Island and those from the South Island, with the notable exception that the northernmost South Island population from Okarito clearly is a sister group to the North Island clade. The latter has strong bootstrap support in the neighbor-joining mtDNA tree. Furthermore, the same two major clades are present in the maximum likelihood tree and the four maximum parsimony trees (data not shown), and the North Island plus Okarito clade and the southern South Island clade are each supported by four derived substitutions. All methods of phylogeny estimation for the allozyme data also group Okarito with the North Island populations. This phylogeographic pattern has never been apparent in morphological characters. In both allozyme and mtDNA trees, the genetic divergence between the two major clades of brown kiwis is roughly equivalent to that between the two sister species of spotted kiwis.

Genetic Population Structure. All but two populations of brown kiwis have different suites of mtDNA haplotypes, which thus diagnose each of them as discrete phylogenetic units. In the northern North Island, two haplotypes are shared between New Plymouth, Little Barrier Island, and Waitangi. However, this may be a consequence of recent management practices because some North Island birds were translocated by man to Little Barrier Island (6) and possibly also to Waitangi. This extreme population structuring is also evident in allozymes (Table 1) where the highest F_{ST} values in birds are observed, rivaling those in highly subdivided populations of small mammals (25, 26). Genetic subdivision at allozyme loci is also evident within these two phylogeographic groups, but F_{ST} is about twice as large among the southern South Island populations (Table 1).

DISCUSSION

Evolution of Population Structure. The most striking finding from our molecular analyses is the extreme population structuring in matrilineal lineages of cytochrome *b*, with virtually every population possessing private alleles. Such a result has never been obtained in any other population study of mtDNA sequences of vertebrates, even when much faster evolving regions of the molecule, such as the control region, are analyzed. Similarly, the allozymes show levels of population subdivision that are equal to the most extreme seen in vertebrates, including cave-dwelling fishes, salamanders, and

Table 2. Characteristics of regional populations of brown kiwis and outgroup spotted kiwis

Character	Population					
	North Island	Okarito	Haast	Fiordland	Stewart Island	Spotted kiwis
Morphology						
Plumage color	Brown	Grey	Rufous	Dark grey	Dark brown	Spotted fawn
Feather tips	Stiff	Soft	Soft	Soft	Soft	Soft
Large tarsal scutes	17	7	4	5	6	6
Facial bristles	Long	Short	Short	Short	Short	Short
Feather lice						
Apterygon	*A. mirum; A. rodericki* on Little Barrier Island	New species	Absent	*A. dumosum*	*A. dumosum*	*A. hintoni**
Rallicola	Absent?	*R. gadowi*	*R. gadowi*	*R. gadowi*	*R. gadowi*	*R. pilgrimi*† and *R. gracilentus**
Allozymes and mtDNA						
Ak-1	A > 0.9; C absent	A = 0.95; B = 0.05	B = 0.17; C = 0.83	A = 1.0	A = 1.0	A > 0.98
Hb-2	A = 1.0	A = 1.0	B = 1.0	B = 1.0	B = 1.0	B = 1.0
Ldh-2	A > 0.8; B and C rare	A = 0.4; C = 0.6	A = 1.0	A = 1.0	A = 1.0	A = 1.0
Cytochrome *b*‡	2–11	12 and 13	14	15 and 16	17–22	1 and 23

*Found only on little spotted kiwi.
†Found only on great spotted kiwi.
‡Numbers refer to haplotypes listed in Fig. 2.

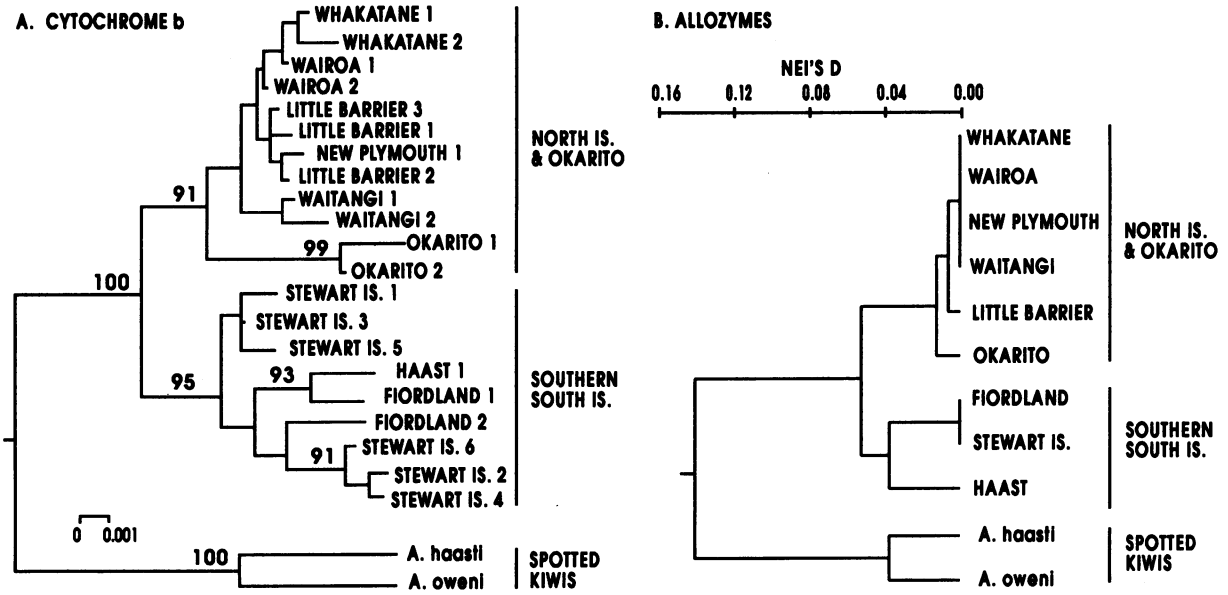

FIG. 3. Molecular phylogenies of kiwis based on a 654-bp fragment of the cytochrome *b* gene of mtDNA (*A*) and 29 allozyme loci (*B*). The mtDNA tree was constructed with the neighbor-joining method on genetic distances corrected for multiple hits with the Kimura two-parameter method. Numbers above branches are the values obtained from 500 replications of the bootstrap. The tree was rooted with an emu (*Dromaius novaehollandiae*) sequence (A.J.B., unpublished data). The allozyme tree was computed with the UPGMA method on Nei's unbiased genetic distance.

small mammals (25–28). In common with kiwis, these taxa have poor dispersal power and are fragmented into small disjunct populations isolated by barriers to gene flow.

Fossil evidence indicates that brown kiwis were once widely distributed over all of New Zealand (6). Range disjunctions must have occurred during the Pleistocene period because large areas of the landmass (particularly in the South Island) were covered by ice. Range fragmentation has been accelerated in the last 1000 years, coincident with the arrival of humans in New Zealand, when vast areas of forest habitat have been destroyed. The hunting of kiwis and the recent introduction of mammalian predators by humans have decimated populations, and this downward trend appears to be continuing today despite conservation efforts. Population sizes are very small in most localities. The Okarito and Haast populations, for example, are currently estimated to be only about 60–100 and 200–300 birds, respectively.

In such small isolated populations, genetic drift will be accentuated, thus accounting for the observed allele frequency shifts at allozyme loci. Because the effective size for mtDNA is only about one-fourth that for nuclear DNA (29), genetic drift alone would be expected to accelerate stochastic lineage extinction within each population (30, 31) and thus explain the remarkable phenomenon of fixation of one or a few private alleles at each locale. Further support for pronounced local differentiation is provided by plumage variation, which is presumably genetically controlled.

Phylogeography, Cryptic Species, and Morphology. The failure of traditional analyses of morphological characters to detect the phylogeographic units revealed in the molecular phylogenies stems from the confusing nature of many of these characters in brown kiwis and lack of outgroup character polarization. However, by mapping them onto the molecular phylogeny, a simple biogeographic hypothesis can explain morphological variation within a phylogenetic framework. The basal southern South Island clade appears to be a remnant of the original ancestral population that colonized northward to Okarito, where they eventually diverged. Birds from this descendent population subsequently invaded North Island and were isolated there after the Cook Strait landbridge was severed by rising water levels in the late Pleistocene. The derived characters (with spotted kiwis as outgroups) of stiff feather tips and greater number of rectangular tarsal scutes of North Island birds can thus, be accounted for by having evolved there in isolation. Conversely, shorter facial bristles and soft feather tips used in historical systematic studies to unite Okarito with the other South Island populations are primitive and, thus, uninformative (Table 2).

The deeper branches in the southern South Island clade in the UPGMA tree for allozymes (Fig. 3*B*) and the neighbor-joining tree for mtDNA contrast with the shallow branches for the Okarito and North Island clade and support the sequential colonization scenario. This hypothesis also explains why the feather louse *Apterygon mirum* is restricted to North Island birds, its probable sister species occurs on Okarito birds, and a more distantly related species occurs on most remaining South Island birds. The louse *R. gadowi* was presumably lost in North Island brown kiwis, with *R. rodericki* apparently being confined to Little Barrier birds (14, 15).

Under the biological species concept, taxonomic assignment of birds in small isolated populations is difficult without the acid test of interbreeding. However, the criterion of reproductive isolation is not particularly informative in kiwis because the brown kiwi and the little spotted kiwi hybridize successfully (A.J.B., unpublished data), despite large differences in morphology, genetics, and ecology. In such cases, the best criterion for species recognition appears to be the monophyly of divergent clades, indicating their discrete phylogenetic histories. The major phylogenetic discontinuity between brown kiwi haplotypes in the mtDNA tree corresponds with the fixed allozyme difference at *Hb*, and the magnitude of genetic divergence in both allozymes and mtDNA equals that between the two species of spotted kiwis (Fig. 3). Thus, on the basis of these criteria, we can reinstate *A. mantelli* and *A. australis* as full species, but with the former including Okarito birds in the South Island. The concordance of the mtDNA and nDNA phylogenies in identifying these monophyletic clades supports this assignment because it indicates that their separation occurred long ago, and thus the trees are very likely good estimates of species phylogeny (8). The detection of morphologically cryptic species in brown kiwis adds yet another mammal-like character to their biology. Irrespective of taxo-

nomic assignments, it is apparent from our study that the appropriate biological unit for conservation of brown kiwis is each population (32). Further molecular analyses of kiwis are urgently required to assist in their conservation management.

Time Scale of Divergence. The kiwis are an ancient lineage judging from the Gondwanaland distribution of the ratites, and they potentially have had 40–80 million years (Myr) to evolve in the isolation of New Zealand. It is therefore of interest to estimate the approximate time scale for population divergence and the evolution of cryptic species in brown kiwis. The percent sequence divergence in the cytochrome *b* gene in geese (33) and shorebirds (A.J.B., unpublished data) is approximately equivalent to that estimated over the whole mtDNA molecule by using restriction fragment length polymorphisms (RFLPs). We can therefore apply the widely used RFLP clock of 2% sequence divergence per 1 Myr to the brown kiwi sequence data and roughly date the phylogeographic splits. The 1.8% sequence divergence between the Okarito population and the basal southern South Island clade suggests that isolation occurred about 900,000 years ago. The 1% sequence divergence between the Okarito and North Island populations dates the evolution of these cryptic species to about 500,000 years ago. Finally, the 0.4% average sequence divergence among North Island populations equates to their isolation in the last 200,000 years. Given the usual caveat about large stochastic errors associated with these estimates, it is nevertheless apparent that genetic subdivision of brown kiwis has evolved recently and coincides with range disjunctions that occurred in the Pleistocene. Thus both vicariance and dispersal have been important in the evolution of intraspecific phylogeography and recent speciation of these flightless birds.

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